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A HILIC-MS/MS Method for the Structural Analysis of Human Alpha-1-Acid Glycoprotein

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Keywords: Alpha-1-acid glycoprotein, biomarker, glycosylation, hydrophilic interaction liquid chromatography, mass spectrometry

1. Introduction

Alpha-1-acid glycoprotein (AGP) is a highly glycosylated acute-phase protein with a molecular weight of 41-43 kDa. The molecule is primarily produced by the liver, however, several extrahepatic tissues have been reported to express AGP under certain pathological states. A vast amount of analytical data indicate that the serum concentration as well as the structure of circulating AGP are changed in such conditions highlighting the biomarker potential of the molecule. Considering that the changes recognized in the glycosylation pattern of AGP take place at the isomeric level, our work has been aimed to develop a high resolution hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) method for the detailed structural analysis of the glycoprotein [1].

2. Methods

Extraction of serum samples was carried out using chloroform-methanol mixture. Separation of AGP from interfering components (mainly albumin) was performed by ion exchange chromatography (IEX) [1]. The oligosaccharide side chains of AGP were then released by Peptide-N-Glycosidase F (PNGase F) enzyme [2]. The released glycans were derivatized with anthranilic acid (AA) [3]. Separation of the sugar constituents was achieved by HILIC using a Phenomenex[®] Luna NH2 column. Mobile phase A was 200 mM ammonium-formate (pH=3.50) and B was acetonitrile. The elution program at flow rate of 0.4 mL/min was as follows: 0-3 min, 70% B; 3-95 min, 70-5% B; 95-110 min, 5% B; 110-111 min, 5-70%B; 111-130

min, 70% B. The glycans eluted were monitored with an orbitrap mass spectrometer operating in negative ion mode. Fragmentation was achieved using higher-energy C-trap dissociation. *Figure 1* summarizes the workflow applied.

3. Results

The extracted ion chromatograms in *Figure 2* show that glycans were eluted in three well-separated peak clusters according to the number of sialic acid residues as follows: 43.49-47.15 min monosialylated; 61.36-75.31 min, bisialylated; 83.46-112.60 min, tri- and tetra-sialylated oligosaccharides. These findings show that the separation of glycans in HILIC is mainly affected by the number of sialic acid residues, while the antennas, fucose units and other structural features such as extra N-acetylglucosamine units have only minor effect on retention. Processing of data acquired in LC-MS experiments resulted in the identification of 102 complex type N-glycan isomers. To the best of our knowledge, this is the highest number ever detected in AGP. Evaluation of MS/MS experiments enabled a reliable characterization of 39 of the most abundant glycan isomers. At a normalized collision energy of 35%, the tandem MS spectra were dominated by Y-, Z- and B-type ion fragments facilitating sequence characterization, however, cross-ring fragmentation resulting A-ions was also observable. Diagnostic fragments appearing on the spectra clearly demonstrated the presence α -2,3 and α -2,6-linked sialic acids in the glycans eluted. Evaluating the MS/MS spectra of baseline separated glycans suggests that the elution order is closely related to sialic acid linkage type, since the increase in the number of α -2,6 linkage resulted in higher reten-

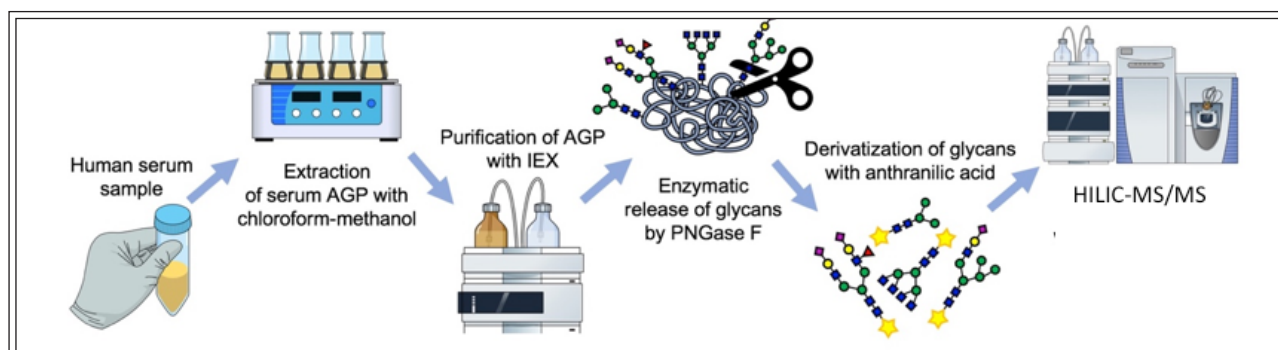


Figure 1 Schematic illustration of the glycoproteomic workflow

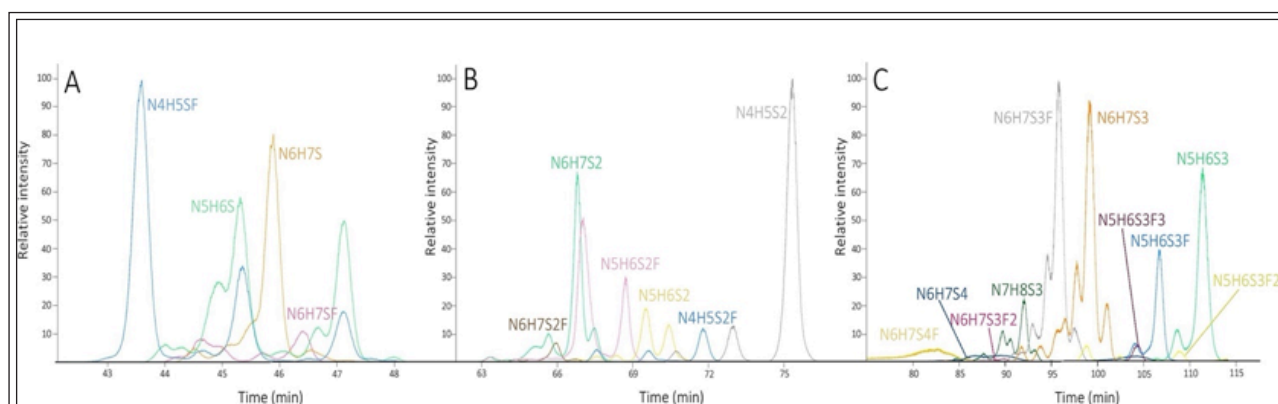


Figure 2 Extracted ion chromatograms of the most abundant mono- (A), bi- (B), tri- and tetrasialylated (C) glycan isomers. N refers to N-acetylglucosamine, H to hexoses (galactose and mannose), S to sialic acid and F to fucose units

tion in HILIC mode. The MS/MS spectra of certain isomers of biantennary glycans N4H5SF and N4H5S2F contained a set of Y (and corresponding Z) ion fragments diagnostic for core fucosylation. Consequently, this study does not support previous findings that AGP is antenna fucosylated exclusively.

4. Conclusions

In this work a high resolution HILIC-MS/MS method was developed for the analysis of AA derivatized N-glycans released from AGP. It enabled the most detailed characterization of the glycan composition of AGP in terms of the number of isomers identified by separating isomers differ only in sialic acid linkage type or in fucose position. These advances can be utilized in glycosylation studies of malignant processes, where many of the changes in the sugar composition of AGP and other glycoproteins can be realized only at the isomeric level. In conclusion, the results presented here may contribute to the development of new biomarkers and the more in-depth understanding of the role of glycosylation in cancerous diseases.

5. Acknowledgements

The work was financed by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the molecular biology thematic programme of Semmelweis University. The research was also supported by the Lendület (Momentum) Program of the Hungarian Academy of Sciences (HAS, MTA) and within the project VEKOP-2.3.3-15-2017-00020 (supported by the European Union and the State of Hungary, co-financed by the European Regional Development Fund).

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